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13. ABSTRACT (Maximum 200 Words)

The overall goal of this proposal is to determine the therapeutic potential of apoptosis-inducing anti-human DR5 and DR4 antibodies, alone or together, in combination with chemotherapeutic drugs with activity against breast cancer, for the treatment of metastatic breast cancer. Aim 1 was to determine the expression profile in human breast cancer cell lines of DR5 and DR4 before and after treatment with anti-DR5 and -DR4 MAb alone, together, and in combination with chemotherapy drugs. Aim 3 was to determine the cytotoxicity of anti-DR5 and -DR4 antibodies against human breast cancer cells alone, together, and in combination with adriamycin or paclitaxel. Aim 4 was to determine the therapeutic efficacy and toxicity of anti-DR5 and -DR4 antibodies against human breast cancer xenografts alone, together, and combined with adriamycin or paclitaxel. All breast cancer cell lines expressed DR5 with TRA-8 reactivity varying from strongly to weakly positive. Four cell lines were sensitive to TRA-8 cytotoxicity with IC_{50} of 17 to 299 ng/ml while other cell lines had weak cytotoxicity or were resistant. *In vivo* studies demonstrated significant inhibition of growth of 2LMP xenografts by TRA-8 treatment alone. TRA-8 alone or in combination with adriamycin, paclitaxel, or radiation produced a significant increase in tumor doubling time compared to any modality alone. Complete tumor regressions occurred in 1/42 untreated animals, 1/54 animals receiving chemotherapy and/or radiation and 28/68 animals receiving TRA-8 alone or TRA-8 combination regimens.

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INTRODUCTION

The purpose of the work carried out during the first year of this project was to evaluate the expression of DR4 and DR5 antigens in a panel of human breast cancer cell lines, and to evaluate the cytotoxicity of TRA-8 (anti-DR5) and 2E12 (anti-DR4) antibodies against the panel of cell lines alone or in combination with adriamycin or paclitaxel. Furthermore, we were interested whether exposure of human breast cancer cells to chemotherapy drugs changed the expression of DR4 or DR5. Most importantly, we wanted to evaluate whether combination treatment of breast cancer xenografts with TRA-8 or 2E12 and chemotherapy resulted in increased therapeutic efficacy compared to antibody or drug treatment alone.

BODY

1. DR5 Expression and TRA-8 Induced Cytotoxicity in Breast Cancer Cell Lines. As illustrated in **Figure 1A**, all nine breast cancer cell lines were DR5 positive with varying degrees of expression from strongly positive (LCC6 and MDA-MB-453) to weakly positive (MDA-MB-468 and SK-BR-3). **Figure 1B** illustrates the TRA-8 induced cytotoxicity of the nine cell lines. Four cell lines were sensitive to TRA-8 induced cytotoxicity with IC_{50} concentrations of 17 to 299 ng/ml (LCC6, 2LMP, MDA-MB-231, MDA-MB-468), while others were quite resistant (DY36T2, BT-474, MDA-MB-453). There was not a good correlation of DR5 expression and degree of TRA-8 induced cytotoxicity as illustrated by cell lines MDA-MB-453 and MDA-MB-468.

TRA-8 effects on chemotherapy-induced cytotoxicity was then examined with adriamycin **(Figure 2A)** and paclitaxel **(Figure 2B)**. An analysis to test for interaction between antibody and drug effects is summarized in **Table 1**. There were no significant synergistic interactions between TRA-8 and paclitaxel, with most of the interactions being additive. Four of nine cell lines fulfilled criteria for a synergistic interaction between TRA-8 and adriamycin. The cell line 2LMP demonstrated good sensitivity to TRA-8, as well as sensitivity to either adriamycin or paclitaxel. This cell line was chosen to explore *in vivo* efficacy of antibody and/or drugs.

Table 1. *In Vitro* Interaction Effects for Combination Treatments

	TRA-8 + Adriamycin		TRA-8 + Paclitaxel	
Cell Line	**Interaction**	**p-value[a]**	**Interaction**	**p-value[a]**
LCC6	Synergistic	<0.001	Additive	0.624
MDA-MB-453	Synergistic	<0.001	No response[c]	0.615
2LMP	Additive	0.153	Additive	0.937
MDA-MB-231	Additive	0.663	Additive	0.064
BT-474	Synergistic	<0.001	ND[b]	0.992
ZR-75-1	Synergistic	0.013	Additive	0.172
DY36T2	ND[b]	0.808	ND[b]	0.798
MDA-MB-468	Additive	0.184	Additive	0.724
SK-BR-3	Additive	0.361	No response[c]	0.871

[a] p-value refers to the significance of the synergistic interaction term. If both TRA-8 and drug effects were significant and the interaction term was significant, then the combination effects were considered synergistic. If the interaction p-value is not < 0.05 then the combination effects were considered additive.

[b] Not determined because the TRA-8 effect was not significant, but the adriamycin/paclitaxel effect was significant.

[c] There was no significant dose response for either agent.

2. DR4 Expression and 2E12 Induced Cytotoxicity in Breast Cancer Cell Lines. All three breast cancer cell lines were DR4 positive **(Figure 3)**. 2E12 effects on adriamycin-induced cytotoxicity was examined *in vitro* for these three cell lines 2LMP, MDA-MB-231, and MDA-MB-453. The test for interaction between 2E12 antibody and drug effects was not significant (p=0.969 and 0.640, respectively). Since the additive effects were significant for both cell lines (all p<0.006), the combination effects were considered additive.

3. *In Vivo* Anti-Tumor Effects of TRA-8 Alone or in Combination with Chemotherapy and/or Radiation. TRA-8 at doses of 200 μg and 600 μg twice a week for 6 doses produced a similar inhibition of tumor growth for well-established 2LMP *s.c.* tumors **(Figure 4)**. In three additional independent experiments, the 200 μg dose/schedule produced statistically significant inhibition of tumor

growth ($p<0.004$, Kruskal-Wallis test on tumor doubling times) compared to untreated controls and this dose and schedule was selected for further studies. **Figure 5** illustrates the effects of TRA-8, adriamycin, or a combination of TRA-8 and adriamycin on anti-tumor efficacy. As compared to untreated controls, therapy with TRA-8 alone or TRA-8 plus adriamycin produced significant inhibition of tumor growth ($p=0.002$ Kruskal-Wallis test), while adriamycin did not differ from controls. The combination of TRA-8 plus adriamycin produced greater growth inhibition than either agent alone ($p=0.002$), as well as significantly more complete regressions of tumor (four) than either agent alone where no complete regressions were seen ($p<0.001$, Fisher exact test). *In vivo* TRA-8 and adriamycin synergism was evaluated using an early growth curve analysis. The interaction term was significant ($p<0.001$) and synergistic. The synergistic interaction was corroborated in a second independent experiment.

The effects of TRA-8 and paclitaxel were studied in this same model with similar observations (**Figure 6**). As compared to untreated controls, TRA-8 and the TRA-8 plus paclitaxel produced significant inhibition of tumor growth ($p<0.001$, Kruskal-Wallis test). Tumor growth in animals treated with TRA-8 plus paclitaxel was significantly different than paclitaxel alone ($p=0.008$) and produced 3/8 complete regressions as compared to none for either agent alone. The effects of the combination of TRA-8 and paclitaxel were additive ($p<0.001$) but not synergistic ($p=0.063$).

Finally, we examined the effects of TRA-8, adriamycin, and ^{60}Co radiation as single agents and in various combinations as illustrated in **Figure 7**. There were significant differences overall with respect to tumor doubling times ($p<0.001$) and multiple comparisons indicated that the triple therapy with TRA-8, adriamycin, and ^{60}Co produced tumor growth inhibition that was significantly different than all other treated groups, while both dual therapy groups (adriamycin plus TRA-8 or ^{60}Co plus TRA-8) were different than either single agent group ($p < 0.001$). The ^{60}Co animals treated with radiation alone did not differ from untreated controls ($p=0.926$). All two-way treatment combinations had significant synergistic effects ($p<0.001$). Complete regressions were seen in 6/8 animals receiving triple therapy and 4 animals did not have tumor recurrence over 180 days of follow-up.

We have begun to evaluate the effect of TRA-8 treatment alone or in combination with chemotherapy against an intermediate sensitivity breast cancer tumor model. LCC6 cells express DR5 and show a lower degree of sensitivity to TRA-8 treatment *in vitro* as compared to 2LMP cells. **Figure 8** shows the tumor growth curve for animals treated with TRA-8 + adriamycin and radiation, TRA-8 + adriamycin + ^{60}Co and adriamycin + ^{60}Co which produced the greatest reduction in time to tumor doubling ($p <0.001$). The anti-tumor effect on the LCC6 tumors was less than that obtained with the more sensitive 2LMP tumor model.

4. Aggregate Analysis of Therapy Effects. The *in vivo* anti-tumor studies were comprised of 166 animals, and we analyzed the tumor doubling times and frequency of complete tumor regression for all animals in each treatment group (**Table 2**). ANOVA analysis for mean tumor doubling times indicated significant differences among treatment groups ($p<0.001$), with multiple comparisons yielding that TRA-8 + paclitaxel, TRA-8 + adriamycin, and TRA-8 + adriamycin + ^{60}Co had significantly longer mean tumor doubling times than any treatment group lacking TRA-8. The addition of TRA-8 to any treatment modality produced a longer tumor doubling time than that modality alone. Similarly, Kruskal-Wallis test on median time to tumor doubling yielded that the medians were significantly different over-all ($p<0.001$). Pair-wise comparisons using Wilcoxin signed-rank test yielded similar patterns for median time to tumor doubling as the ANOVA multiple comparisons. This analysis underestimates the growth inhibition produced by the most effective treatments in that groups that did not reach a doubling of tumor by the end of the experiment were assigned the experiment termination day. **Table 2** also provides the frequency of complete regression of tumor and the frequency of persistence of that regression to the end of the experiment. There were no complete regressions of tumor seen in animals treated with either chemotherapy regimen or radiation attesting to the well-established tumor growth and tumor aggressiveness. From Fisher's exact test, there were significant differences in the frequency of tumor complete regressions between treatment groups ($p<0.001$). Thirty of 166 animals achieved complete regression, and 28 of these received TRA-8 alone or in combination with other modalities. Complete regression occurred in 1/42 control animals: 1/54 animals receiving chemotherapy, radiation, or a combination; and 28/68 of TRA-8 alone or TRA-8 combination regimens. The TRA-8 treated groups had a significantly ($p<0.001$) greater frequency of complete regression. Similarly, 14/68 animals receiving TRA-8 or TRA-8 combinations did not have tumor re-growth compared to 1/42 controls and 0/52 animals treated with chemotherapy and/or radiation. The relapse-free regressions had observation periods of 99 to 171 days (146 ± 24 days).

Table 2. Aggregate Results of Doubling Time and Complete Regression of 2LMP Tumors

Treatment	# of Animals	Tumor Doubling Time (days) (mean/median)	Complete Regressions		
			Total (%)	No relapse (%)	Mean Observation Period (days)
Untreated Controls	44 (42)[a]	12/8	1 (2%)	1 (2%)	177
^{60}Co	8 (7)	14/10	0	0	186
Adriamycin	31 (28)	17/18	0	0	197
Paclitaxel	7 (5)	25/20	0	0	-
Adriamycin + ^{60}Co	8 (8)	39/36	1 (13%)	0	197
TRA-8	30 (26)	47/23	6 (20%)	5 (17%)	159
TRA-8 + ^{60}Co	8 (8)	65/50	3 (38%)	1 (13%)	186
TRA-8 + Paclitaxel	8 (8)	71/62	3 (38%)	1 (13%)	148
TRA-8 + Adriamycin	14 (12)	81/64	10 (71%)	3 (21%)	185
TRA-8 + Adriamycin + ^{60}Co	8 (6)	>140/179	6 (75%)	4 (50%)	192

[a] The numbers in parentheses are the number of uncensored animals.

5. Apoptosis in Treated Tumors. The induction of apoptosis in 2LMP xenografts following treatment with TRA-8, adriamycin, paclitaxel, TRA-8 + adriamycin, and TRA-8 + paclitaxel was assessed using the TUNEL technique (**Figure 9**). In untreated animals, tumors had 4% stained cells (1% intense), while treatment with adriamycin or paclitaxel had 8% (6% intense) and 7% (2% intense) stained cells. Animals treated with TRA-8 alone had striking apoptosis with 25% (15% intense) stained cells. TRA-8 plus adriamycin had 28% (22% intense) and TRA-8 plus paclitaxel had 26% (12% intense) stained cells.

6. Effect of a Second Course of Treatment with TRA-8 and Adriamycin. We initiated an experiment to determine whether a second course of treatment with TRA-8 and adriamycin would inhibit 2LMP *s.c.* breast cancer xenograft beyond that achieved with a single course of treatment. TRA-8 (200 μg) was injected *i.p.* into a group of athymic nude mice on day 8 after tumor cell injection, and additional doses of 200 μg TRA-8 were administered on days 12, 15, 19, 22, and 26. Adriamycin (6 mg/kg) was injected *i.v.* on days 9, 13, and 17. A second group of 8 animals received a second course of treatment with TRA-8 and adriamycin starting at 7 days after the last injection of TRA-8 in the first course of treatment (*i.e.* 200 μg TRA-8 injected *i.p.* on days 33, 36, 40, 43, 47, and 50 and 6 mg/kg adriamycin *i.v.* on days 34, 38, and 42). Other groups of animals received 1 or 2 courses of TRA-8 alone, adriamycin alone, or were untreated. The results of this ongoing study are shown in **Figure 10**. There were 3/8 complete regressions in the group treated with two courses of TRA-8, 1/8 in the group receiving one course of TRA-8 + adriamycin, and 2/8 in the group treated with two courses of TRA-8 + adriamycin. The results indicate that the growth of 2LMP xenografts was further inhibited by a second course of treatment with TRA-8 alone, adriamycin alone, or TRA-8 plus adriamycin, and show that the tumors that survive the first course of therapy with TRA-8, adriamycin, or the combination of these two agents are responsive to a second course of therapy with each agent. In addition, the most effective treatment was combined treatment with TRA-8 and adriamycin. Furthermore, the tumors appear to continue to express DR5, since two courses of TRA-8 inhibited tumor growth to a greater extent than a single course of treatment.

7. Anti-Tumor Efficacy of 2E12 Alone or in Combination with Adriamycin. We examined the effect of 2E12 alone and in combination with adriamycin in athymic nude mice bearing 2LMP breast cancer xenografts (**Figure 11** and **Table 3**). Seven animals were untreated controls, 8 animals were treated with adriamycin alone, 8 animals were treated with 2E12 alone and 8 were treated with the adriamycin + 2E12 combination. The median times to tumor doubling are given for each therapy group in **Table 3**. From the Kruskal-Wallis test, there were significant differences in growth inhibition in terms of time to tumor doubling ($p<0.001$). Multiple comparisons indicated that 2E12 in combination with adriamycin significantly increased the time to tumor doubling over either therapy alone and both single therapies had significant increases over controls, but the single therapy groups were not significantly different from each other. Moreover, 2E12 in combination with adriamycin produced significant increases in tumor regression and recurrence-free tumor regression rates ($p = 0.008$ and $p=0.045$, respectively), as compared to either agent alone or untreated controls. While no tumor regressions occurred in the untreated controls or the single therapy groups, 4 out of 8 tumors in the adriamycin + 2E12 group regressed. On average these

regression occurred within 20 days after start of therapy. Three out of the 4 regressions were recurrence-free, with an average follow-up period of 41 ± 13 days. The treatment groups were also compared with respect to growth inhibition. From ANOVA, there were significant differences in growth inhibition between the treatment groups ($p<0.001$), with multiple comparisons indicating that all treatment groups had significantly inhibited tumor growth as compared to controls (mean time to tumor doubling was 7 days). The single modality, 2E12 and adriamycin alone mean time to tumor doubling (29 and 21 days, respectively), were not significantly different from one another but achieved significantly less growth inhibition than the 2E12 + adriamycin combination (mean time to tumor doubling was over 97 days). Therefore, adriamycin + 2E12 treatment combination produced a significant amount of tumor growth inhibition, as compared to either therapy alone or no treatment.

Table 3. Anti-tumor Efficacy for 2E12 alone and in Combination with Adriamycin

Treatment	# of Animals	Tumor Doubling Time (mean/median)	Complete Regressions		
			Total (%)	No relapse (%)	Mean Recurrence
Untreated Controls	7	7/7	0	0	-
Adriamycin	8	21/23	0	0	-
2E12	8	29/23	0	0	-
2E12 + Adriamycin	8	> 97/65	4 (50%)	3 (38%)	5 (n=1)

8. Combination Treatment with TRA-8 plus 2E12 With or Without Adriamycin in Nude Mice Bearing 2LMP Xenografts. We next examined the effect of combining TRA-8 and 2E12 (same dose and schedule) with or without adriamycin (**Figure 12** and **Table 4**). Thirty-two animals were randomly assigned into groups of 8 animals, untreated controls, adriamycin, 2E12 + TRA-8, and 2E12 + TRA-8 + adriamycin. The mean and median time to tumor doubling for each group are given in **Table 4**. From the Kruskal-Wallis test, there were significant differences in the tumor growth inhibition with respect to tumor doubling times, on average ($p<0.001$). Multiple comparisons indicated that both the 2E12 + TRA-8 and 2E12 + TRA-8 + adriamycin therapy groups significantly increased the time to tumor doubling over all other groups, but these two regimens were not significantly different from each other. Adriamycin treatment was not significantly different than the controls. Moreover, the combination of the two antibodies produced significant increases in tumor regression and recurrence-free regression rates, as compared to untreated controls and single-agent therapy groups ($p < 0.001$). No tumor regressions occurred in the untreated controls or in the adriamycin treated animals, but a striking number, 7 out of 8 of the 2E12 + TRA-8 and 8 out of 8 of the 2E12 + TRA-8 + adriamycin treated animals, had complete tumor regressions, occurring on average just 6 days ± 1.5 days after start of therapy. Even more striking is the fact that 7 out of 8 of the 2E12 + TRA-8 + adriamycin tumor regressions were recurrence-free, with an average follow up of 176 ± 2 days. The treatment groups were also compared with respect to growth inhibition. From ANOVA, there were significant differences in growth inhibition between the treatment groups ($p<0.001$), with multiple comparisons indicating that the 2E12 + TRA-8 and 2E12 + TRA-8 + adriamycin treated animals (mean times to tumor doubling were over 131 and 158 days, respectively) significantly inhibited tumor growth as compared to the untreated controls and adriamycin treated animals (mean time to tumor doubling, 9 and 13 days, respectively). Although not statistically different from 2E12 + TRA-8, the 2E12 + TRA-8 + adriamycin treated animals did achieve the greatest observed inhibition of tumor growth with 100% complete tumor regressions and 88% relapse-free. In addition, this finding for 2E12 + TRA-8 + adriamycin demonstrated improvement over the results from the experiment involving the TRA-8 and adriamycin combinations, described above .

Table 4. Anti-tumor Efficacy for 2E12 and TRA-8 in Combination with Adriamycin

Treatment	# of Animals	Tumor Doubling Time (mean/median)	Complete Regressions		
			Total (%)	No relapse (%)	Mean Recurrence
Untreated Controls	8	9/8	0	0	-
Adriamycin	8	13/14	0	0	-
2E12 + TRA-8	8	> 131/182	7 (88%)	5 (63%)	22 (n=2)
2E12 + TRA-8 + Adriamycin	8	> 158/185	8 (100%)	7 (88%)	97 (n=1)

9. Upregulation of DR5 by Adriamycin or Radiation in LCC6 Cells. Figure 13 shows that exposure of LCC6 breast cancer cells to adriamycin or radiation results in increased expression of DR5.

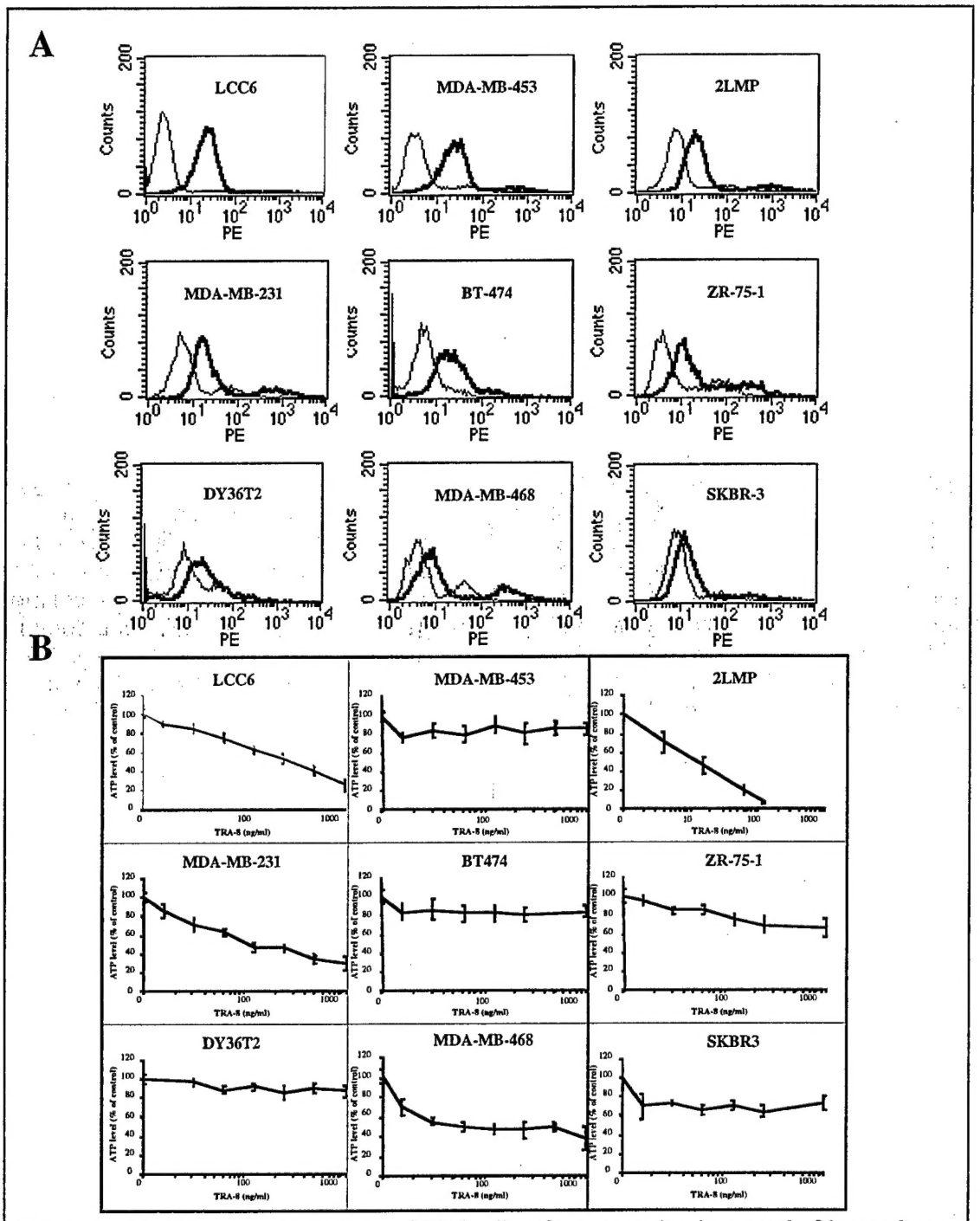

Figure 1. **A**) Flow cytometry analysis of DR5 cell surface expression in a panel of human breast cancer cell lines. Breast cancer cells were harvested using EDTA and stained with 10 μg/ml TRA-8 mAb for 1 h at 4°C followed by PE-conjugated goat anti-mouse IgG1, then analyzed using FACScan and CellQuest software. Thick histograms indicate TRA-8 staining and thin histograms indicate incubation with mouse IgG1 isotype control antibody. **B**) Cytotoxicity of TRA-8 to human breast cancer cell lines. Cells were trypsinized and replated at a density of 1,000 cells/well in a 96-well plate. TRA-8 antibody was added after plating cells, and incubated for 24 h at 37°C. Cell viability was assessed 24 h after TRA-8 addition using the ATPLite assay. ATP levels are reported relative to untreated control cells as the mean and SE from 2-3 independent experiments, each done in triplicate.

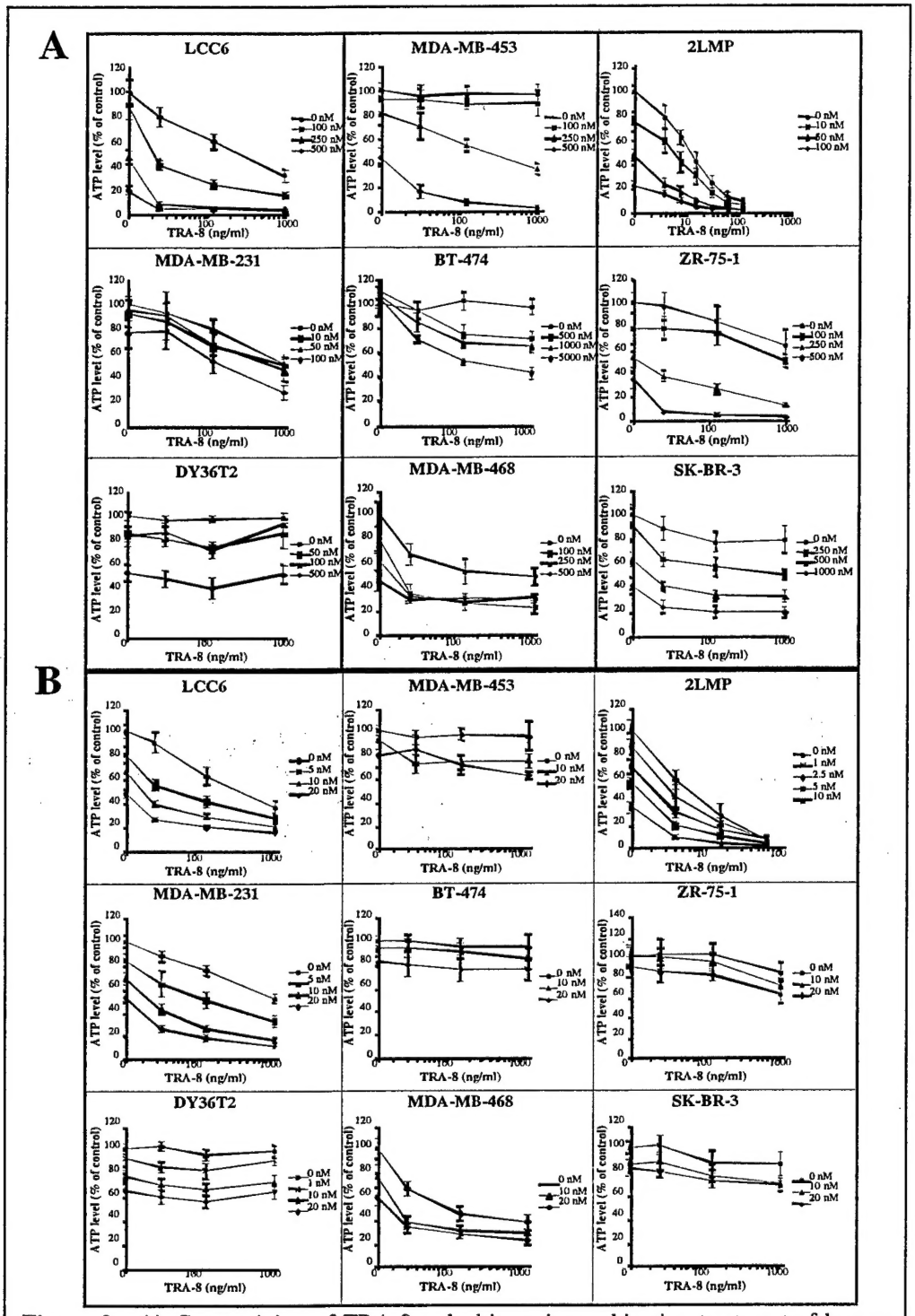

Figure 2. **A)** Cytotoxicity of TRA-8 and adriamycin combination treatment of human breast cancer cell lines. Cells (1,000/well) were exposed to various concentrations of adriamycin for 24 h at 37°C beginning 24 h after plating cells. TRA-8 was added 24 h after adriamycin addition, and ATP levels were determined 24 h later. Values represent the mean and SE of triplicate determinations from 2-4 independent experiments each done in triplicate, and are reported relative to untreated control cells. **B)** Cytotoxicity of TRA-8 and paclitaxel combination treatment of human breast cancer cell lines. Cells (1,000/well) were exposed to various concentrations of paclitaxel for 24 h at 37°C beginning 24 h after plating cells. TRA-8 was added 24 h after paclitaxel addition, and ATP levels were determined 24 h later. Values represent the mean and SE of triplicate determinations from 2-4 independent experiments each done in triplicate, and are reported relative to untreated control cells.

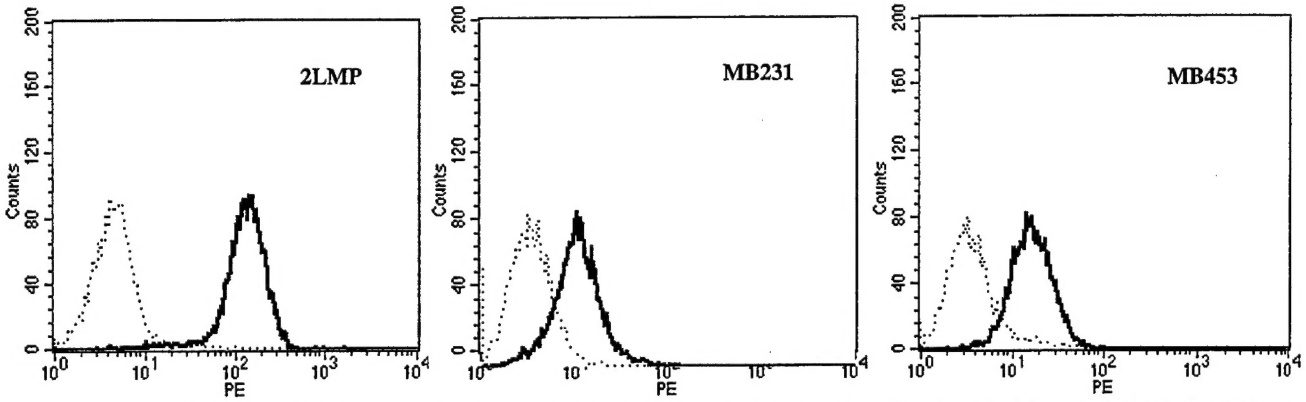

Figure 3. Binding of 2E12 to human breast cancer cell lines. Cells were incubated with 10 μg/ml TRA-8, 2E12, or control IgG1 antibody for 1 h at 4°C followed by PE-conjugated anti-mouse IgG1. The thick histograms indicate cells incubated with TRA-8 or 2E12 whereas the thin histograms indicate incubation with control IgG1 antibody.

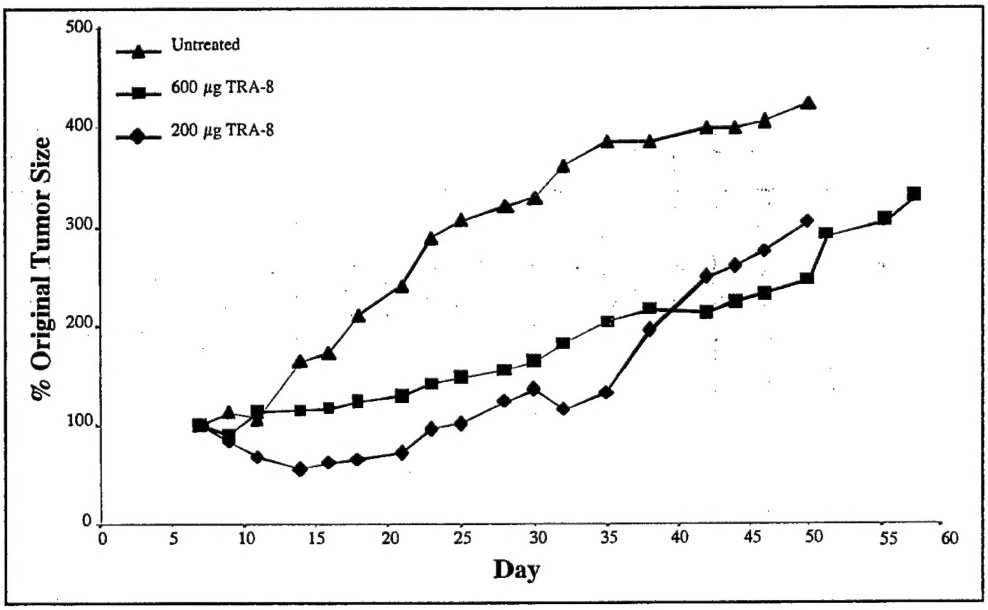

Figure 4. The effect of TRA-8 on tumor growth in athymic nude mice bearing established 2LMP human breast cancer xenografts. 2LMP cells ($3x10^7$) were injected *s.c.* on day 0. Two groups of mice were injected *i.p.* with 200 μg or 600 μg TRA-8 on days 7, 10, 14, 17, 21, and 24. One group of mice received no antibody. The data represent the average change in tumor size (product of two diameters) relative to size on day 7 (n=8 mice/ group).

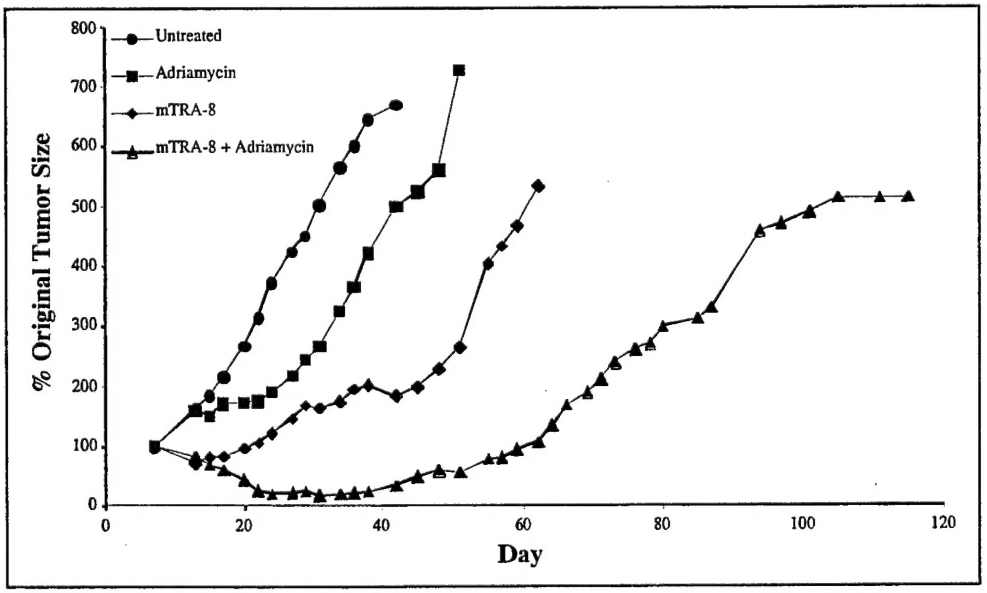

Figure 5. The effect of TRA-8 and adriamycin on tumor growth in athymic nude mice bearing breast cancer xenografts. 2LMP cells ($3x10^7$) were injected *s.c.* into athymic nude mice on day 0. Two groups of mice were injected *i.p.* with 200 μg TRA-8 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received *i.v.* adriamycin (6 mg/kg) on days 8, 12, and 16. One group of mice received no antibody. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 7 (n=6-8 mice/ group).

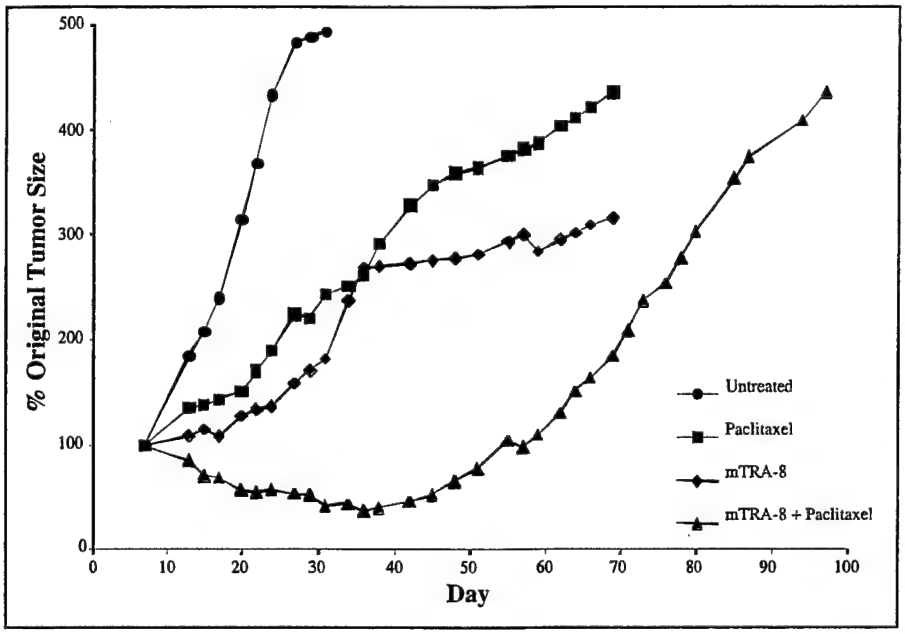

Figure 6. The effect of TRA-8 and paclitaxel in athymic nude mice bearing breast cancer xenografts. 2LMP cells ($3x10^7$) were injected *s.c.* into athymic nude mice on day 0. Two groups of mice were injected *i.p.* with 200 μg TRA-8 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received *i.v.* paclitaxel (20 mg/kg) on days 8, 12, 16, 20, and 24. One group of mice received no antibody. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 7 (n=8 mice/group).

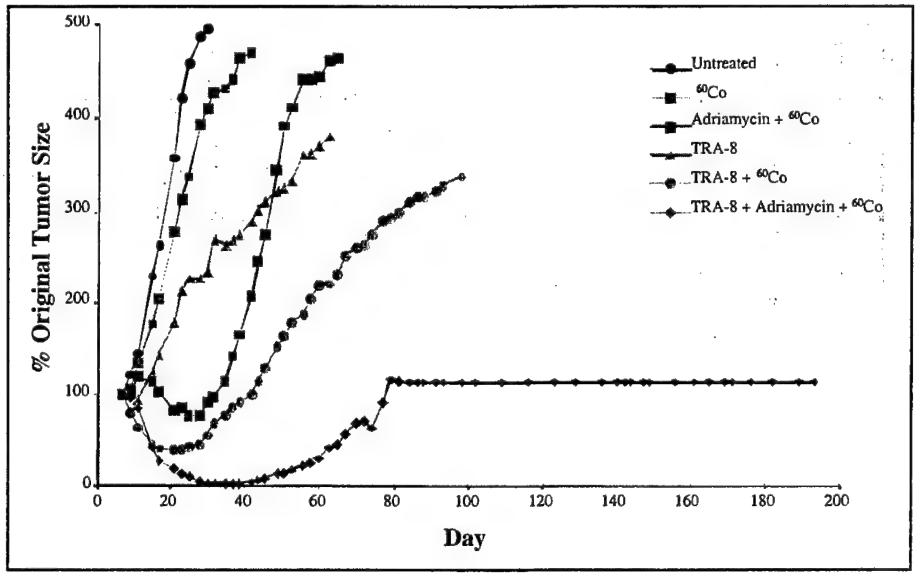

Figure 7. The effect of TRA-8, adriamycin, and ^{60}Co radiation on tumor growth in athymic nude mice bearing breast cancer xenografts. 2LMP cells ($3x10^7$) were injected *s.c.* into athymic nude mice on day 0. Three groups of mice were injected *i.p.* with 200 μg TRA-8 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received *i.v.* adriamycin (6 mg/kg) on days 8, 12, and 16. Four groups of mice received 3 Gy ^{60}Co radiation on days 9 and 17. One group of mice received no antibody. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 7 (n=8 mice/ group).

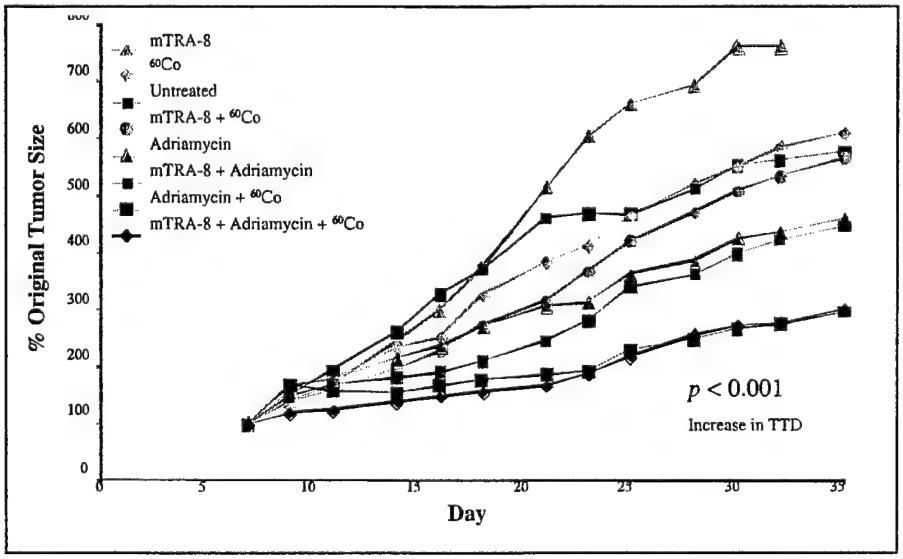

Figure 8. The effect of TRA-8, adriamycin, and radiation on growth of LCC6 breast cancer xenografts. See **Figure 7** legend for details.

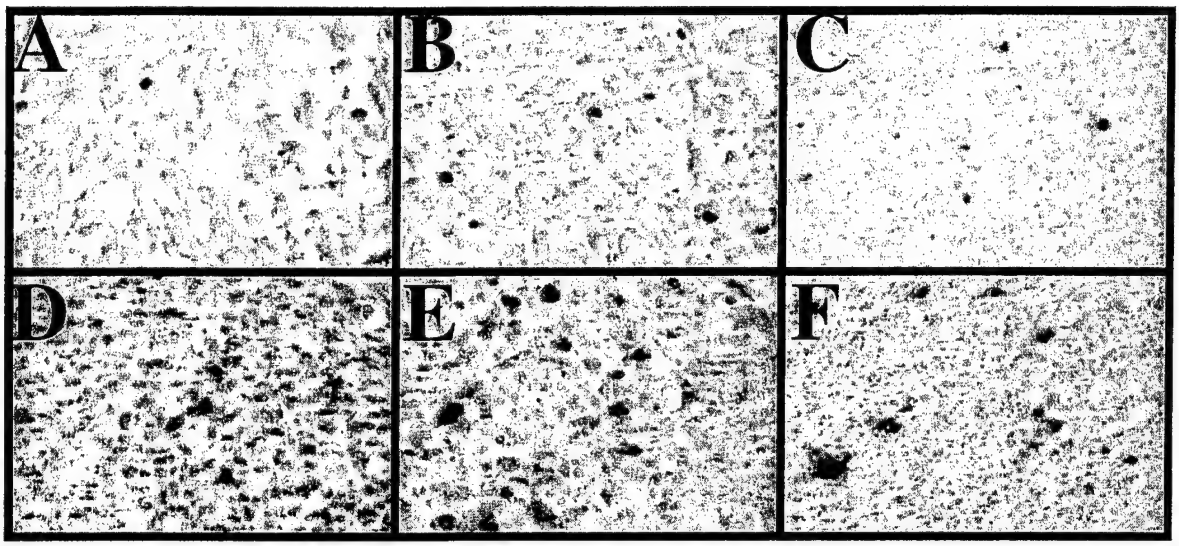

Figure 9. **A)** Demonstrates an apparently viable area of a 2LMP tumor xenograft from an untreated nude mouse; **B)** demonstrates a viable area of a 2LMP xenograft tumor from an animal treated with adriamycin only. Like the untreated tumor, few cells exhibited apoptosis (dark brown); **C)** demonstrates a viable area of a 2LMP xenograft tumor from an animal treated with paclitaxel only. Like the untreated tumor, few cells exhibited apoptosis (dark brown); **D)** demonstrates a characteristic area of tumors from animals treated only with TRA-8 antibody at a dose of 100 μg administered twice with a 3 day interval between injections. A high percentage of cells demonstrate apoptosis; **E)** demonstrates a characteristic area of tumors from animals treated with the TRA-8 antibody plus adriamycin. Just as in tumors treated only with TRA-8, a high percentage of cells are undergoing apoptosis; **F)** demonstrates a characteristic area of tumors from animals treated with the TRA-8 antibody plus paclitaxel. Just as in tumors treated only with TRA-8, a high percentage of cells are undergoing apoptosis. Magnification 400X.

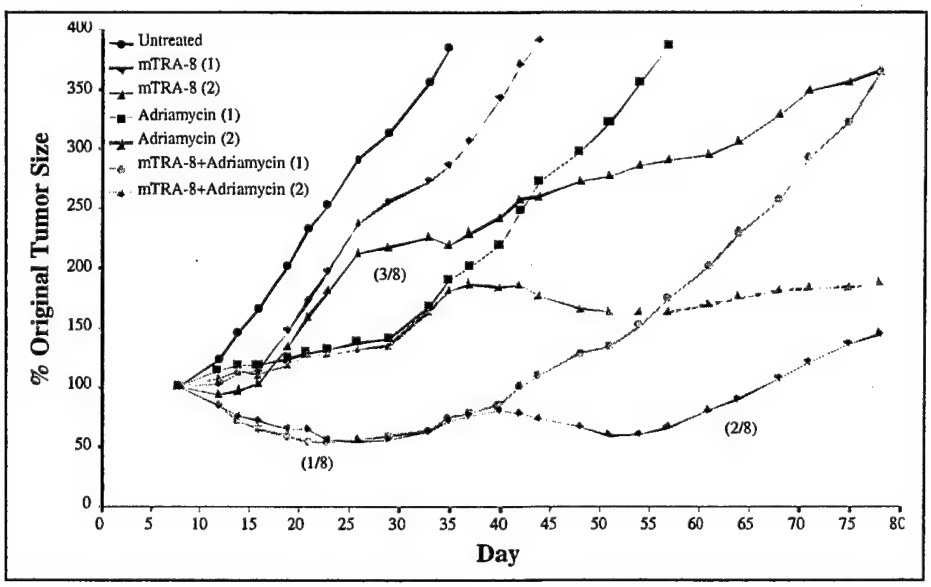

Figure 10. Treatment of 2LMP *s.c.* xenografts with one or two courses of TRA-8 and adriamycin. Groups of 8 mice were injected *s.c.* with $3x10^7$ 2LMP cells on day 0. Course 1 began on day 8 and lasted until day 26. Four groups of mice were injected *i.v.* with 200 μg TRA-8 on days 8, 12, 15, 19, 22, and 26. Four groups of mice were injected *i.v.* with 6 mg/kg adriamycin on days 9, 13, and 17. Course 2 began on day 33 and ended on day 50. Groups of mice previously treated with TRA-8 received 200 μg TRA-8 *i.v.* on days 33, 36, 40, 43, 47, and 50. Groups of mice previously treated with CPT-11 received 6 mg/kg CPT-11 *i.v.* on days 34, 38, and 42. Data are expressed as the average change in tumor size (surface area equal to product of two largest diameters) relative to size on day 8. Complete regressions are shown in parentheses. Dashed line indicates only 3/8 surviving mice.

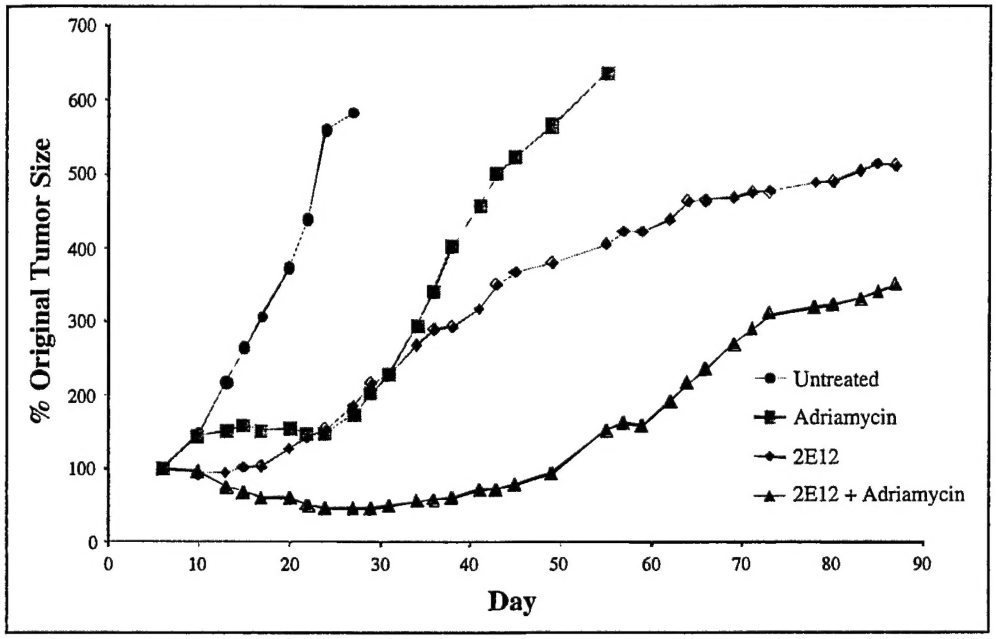

Figure 11. The effect of 2E12 and adriamycin in athymic nude mice bearing breast cancer xenografts. 2LMP cells ($3x10^7$) were injected *s.c.* into athymic nude mice on day 0. Two groups of mice were injected *i.p.* with 200 μg 2E12 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received *i.v.* adriamycin (6 mg/kg) on days 8, 12, and 16. One group of mice received no antibody. Data are expressed as the average change in tumor size relative to size on day 7 (n=8 mice/ group).

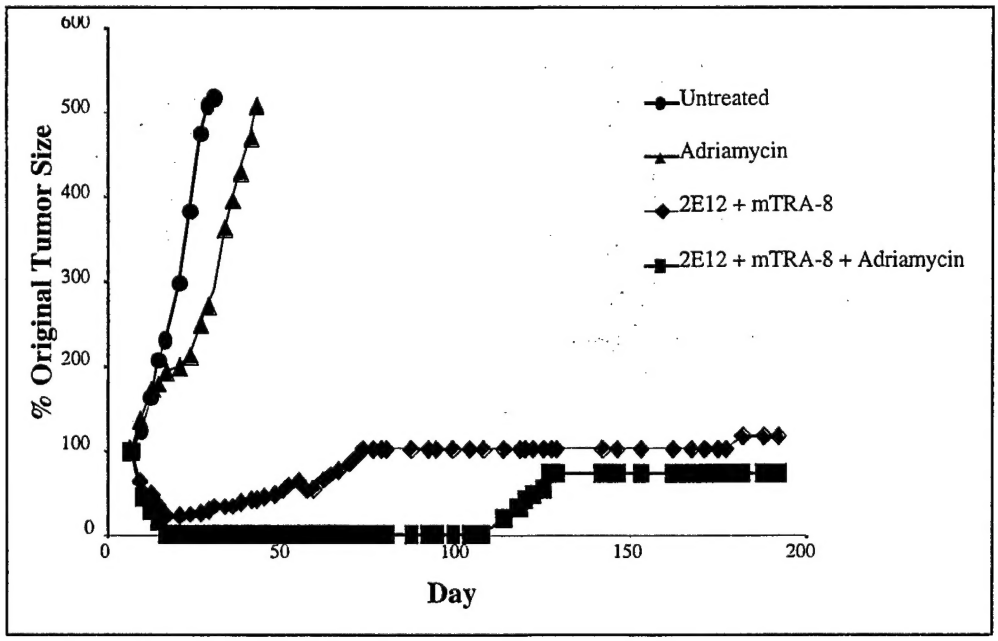

Figure 12. The effect of TRA-8, 2E12 and adriamycin in athymic nude mice bearing breast cancer xenografts. 2LMP cells ($3x10^7$) were injected *s.c.* into athymic nude mice on day 0. Two groups of mice were injected *i.p.* with 200 μg TRA-8 and 2E12 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received *i.v.* adriamycin (6 mg/kg) on days 8, 12, and 16. One group of mice received no antibody. Data are expressed as the average change in tumor size relative to size on day 7 (n=8 mice/ group).

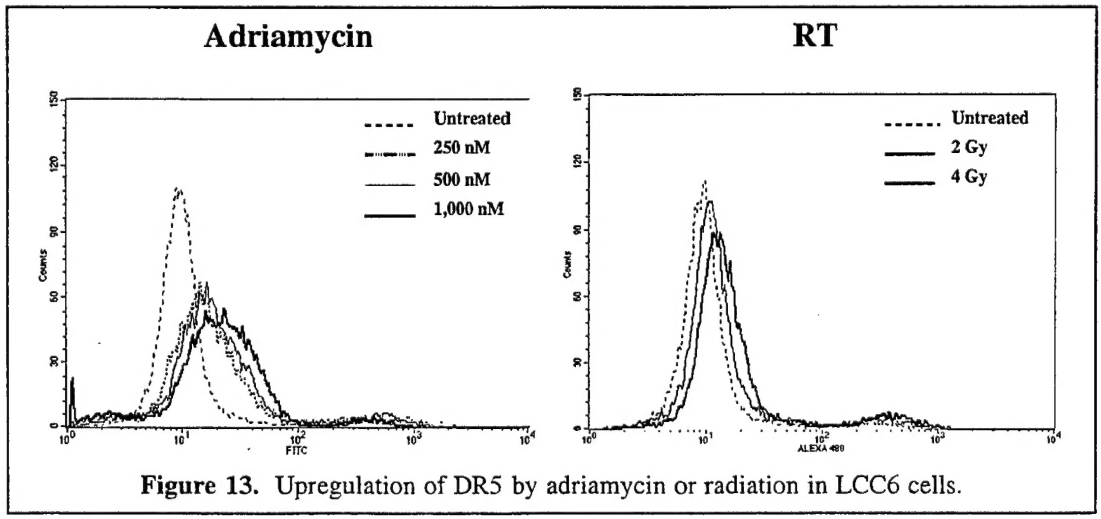

Figure 13. Upregulation of DR5 by adriamycin or radiation in LCC6 cells.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated DR4 and DR5 expression on a large panel of human breast cancer cell lines.
- Demonstrated variable cytotoxicity of human breast cancer cell lines to TRA-8 and 2E12 alone or in combination with adriamycin or paclitaxel.
- Showed that DR5 cell surface expression is increased by exposure to adriamycin or radiation *in vitro*.
- Demonstrated increased antitumor efficacy *in vivo* of TRA-8 and 2E12 in combination with adriamycin, paclitaxel, and radiation.

REPORTABLE OUTCOMES

Developed two monoclonal antibodies that interact synergistically with chemotherapy drugs and radiation.

CONCLUSIONS

These studies indicate that targeting either DR4 or DR5 can produce anti-tumor effects, that both monoclonal antibodies can enhance drug (adriamycin) anti-tumor effects and that the combination of both antibodies has a striking anti-tumor efficacy. TRA-8 was found to react with all nine breast cancer cell lines examined and these cell lines were found to have a range of *in vitro* sensitivity to antibody mediated cytotoxicity similar to the variability that has been reported with TRAIL. Co-incubation of TRA-8 and adriamycin or paclitaxel produced enhancement of cytotoxicity compared to either agent alone in TRA-8 sensitive breast cancer cell lines. The enhancement was synergistic in certain cell lines and additive in others. For the *in vivo* studies, we utilized the 2LMP breast cancer cell line that was developed as a more aggressive sub-clone derived from MDA-MB-231. This cell line had moderate expression of DR5 and was sensitive to TRA-8 induced cytotoxicity *in vitro*. The 2LMP cell line had a dose dependent cytotoxicity with either adriamycin or paclitaxel, and the combination of TRA-8 and adriamycin or paclitaxel produced additive enhancement of cytotoxicity. Neither adriamycin nor paclitaxel produced significant growth inhibition compared to controls, while producing striking tumor inhibition and tumor regression when combined with TRA-8. The combination of adriamycin and TRA-8 fulfilled the criteria for *in vivo* synergism ($p < 0.001$) and produced 4 out of 8 complete regressions of tumor. The combination of paclitaxel and TRA-8 produced similar effects, although the interaction met criteria for additive effects and included 3 of 8 complete regressions. Forty-one percent of animals receiving TRA-8 alone or in combination with chemotherapy and/or radiation had complete regressions (28 of 68 animals). Further, 21% of these animals had no evidence of tumor recurrence over 148-192 days of observation. The TRA-8 regimens complete regression rate, rate of recurrence-free complete regressions, and effects on tumor doubling time were all statistically different than single agents or non-TRA-8 combinations.

Statement of Work

SPECIFIC AIM #1. To determine the expression profile in human breast cancer cell lines of DR5 and DR4 before and after treatment with anti-DR5 and -DR4 MAb alone, together, and in combination with chemotherapy drugs.

Task 1: *Months 1-3. To determine cell surface expression of DR5 and DR4 in untreated breast cancer cells by flow cytometry analysis.*

Task 2: *Months 1-6. To determine whether there is a change in DR5 and DR4 expression in human breast cancer cells after exposure to anti-DR5 and -DR4 MAb alone or in combination with chemotherapy drugs.*

SPECIFIC AIM #2. To determine the expression profile of DR5 and DR4 during the progression of breast cancer.

Task 1: *Months 6-36. To determine DR5 and DR4 expression in breast cancer tissues by immunohistochemistry staining.*

Task 2: *Months 6-36. To determine whether there is a correlation of increased DR5 and DR4 expression with the progression of breast cancer and other tumor markers.*

SPECIFIC AIM #3. To determine the cytotoxicity of anti-DR5 and -DR4 antibodies against human breast cancer cells alone, together, and in combination with adriamycin or paclitaxel.

Task 1: *Months 6-18. To examine DR5 and DR4 positive breast cancer cell lines for their susceptibility to anti-DR5 and -DR4 antibody-mediated apoptosis in the presence or absence of chemotherapy drugs.*

Task 2: *Months 12-24. To determine the effect of timing of drug exposure, before, at the same time, or following antibody exposure to identify the optimum regimen for the induction of apoptosis.*

SPECIFIC AIM #4. To determine the therapeutic efficacy and toxicity of anti-DR5 and -DR4 antibodies against human breast cancer xenografts alone, together, and in combination with adriamycin or paclitaxel.

Task 1: *Months 6-24. To examine the in vivo therapeutic potential of TRA-8 and anti-DR4 antibodies alone, together, and in combination with chemotherapy drugs in nude mice with localized breast cancer xenografts.*

Task 2: Months 18-26. To examine the in vivo therapeutic potential of TRA-8 and anti-DR4 antibodies alone, together, and in combination with chemotherapy drugs in nude mice with metastatic breast cancer xenografts.

The progress during the first year matched the previously submitted Statement of Work closely. Specific Aim 2 has been deferred to year 02. The goals for year 02 remain as previously stated.

ABBREVIATIONS

DR4	TRAIL death receptor 4
DR5	TRAIL death receptor 5
MAb	Monoclonal antibody

REFERENCES

1. Buchsbaum DJ, Zhou T, Grizzle WE, Oliver PG, Hammond CJ, Carpenter M, LoBuglio AF: Antitumor efficacy of TRA-8 anti-DR5 monoclonal antibody alone or in combination with chemotherapy and/or radiation therapy in a human breast cancer model. *Clin Cancer Res* In Press 2003.
2. Ohtsuka T, Buchsbaum D, Oliver P, Makhija S, Kimberly R, Zhou T: Synergistic induction of tumor cell apoptosis by death receptor antibody and chemotherapy agent through JNK/p38 and mitochondrial death pathway. *Oncogene* 22: 2034-2044, 2003.

Figure 9